maintain the mature chondrocyte phenotype avoiding terminal differentiation towards hypertrophy represents a major issue and is the objective of this study.

Methods: Bone marrow-derived murine MSCs were induced to differentiate towards chondrocytes using the micropellet culture technique in presence of Wnt-6 containing conditioned medium (CM). CM was obtained after incubation of a chondrogenic medium consisting of DMEM supplemented with ITS, proline, ascorbic acid and sodium pyruvate for 48h on confluent NIH-3T3 cells stably transfected to secrete Wnt-6. As controls, CM from NIH-3T3 cells or BMP-2 containing medium was used. Similar conditions were used to obtain CM with osteogenic or adipogenic media. After 21 days, quantitative RT-PCR was performed on total RNA to detect the expression of markers specific for each lineage and staining specific for proteoglycans, mineralization or lipid droplets was performed. Western blotting with anti-β-catenin, anti-JNK or anti-PKC antibodies was performed after migration and transfer on nitrocellulose membranes of 20 μg total proteins extracted from pellets after a time course exposure to the different CM.

Results: Pellet culture of murine MSCs in presence of NIH-derived CM or chondrogenic medium alone did not up-regulate the chondrocytic markers. On the contrary, Wnt-6 containing CM was sufficient to induce the differentiation of MSCs into chondrocytes as shown by the induction of collagen type IIB, aggrecan and COMP and a positive staining for proteoglycans. The expression levels of the transcripts were lower than those induced by BMP-2. However, contrary to BMP-2, we observed the lack of induction of the hypertrophic markers collagen type X and alkaline phosphatase when MSCs were cultured in Wnt-6 CM. Interestingly, in osteogenic or adipogenic conditions, MSCs did not up-regulate the markers specific for osteoblasts or adipocytes and rather decreased their expression level. The up-regulation of chondrocytic markers by Wnt-6 was associated with a lack of induction of the β-catenin or JNK pathways and preliminary results suggest that PKC signalling may be induced.

Conclusions: Our results suggest that Wnt-6 is one new chondrogenic factor sufficient to specifically induce the generation of chondrocytes and inhibiting their terminal differentiation. Preliminary results suggest that Wnt-6 might induce the PKC-dependent pathway to activate the chondrocyte-specific genes.

163

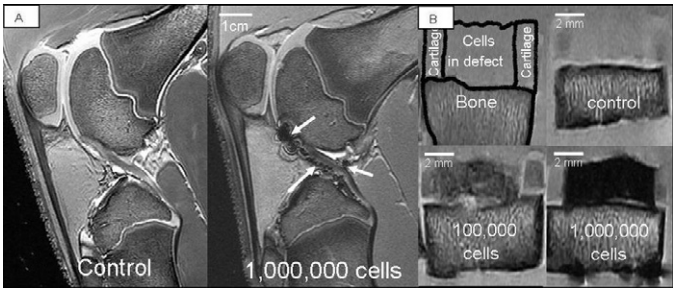
CELL TRACKING FOR CARTILAGE REPAIR USING SUPERPARAMAGNETIC IRON OXIDES: CLINICAL POTENTIAL

G.M. van Buul, G. Kotek, P.A. Wielopolski, E. Farrell, P.K. Bos, H. Weinans, J.A. Verhaar, G.P. Krestin, M.R. Bernsen, **G.J. van Osch**
Erasmus MC, Rotterdam, Netherlands

Purpose: Human bone marrow stromal cells (hBMSCs) are experimentally being used in patients as a cell-based therapy for cartilage repair. To verify the safety and efficacy of such approaches it is necessary to determine the fate of these implanted cells. Cell labeling using superparamagnetic iron oxides (SPIOs) enables non-invasive in vivo cell tracking by MRI, and has already been used in a clinical setting in various fields. In this study we describe a major step towards application of SPIO-labeling for cell tracking in clinical cell-based cartilage repair approaches. We investigated the safety, intra-articular MRI traceability and the possibility of SPIO re-uptake of this cell tracking technique.

Methods: *Safety:* hBMSCs from three donors were labeled in triplicate samples with ferumoxides (Endorem®)-protamine sulphate complexes at doses ranging from 0 - 250 μg iron/ml. After incubation for 24 hours, cell viability was assessed using a trypan blue exclusion assay. Subsequently, metabolic cell activity was quantified using the AlamarBlue® assay up to seven days after labeling. Chondrogenic capacity of hBMSCs labeled with 100 μg/ml SPIO was evaluated using thionine staining and collagen type II immunohistochemistry.
Intra-articular imaging: SPIO-labeled hBMSCs (100,000 to 5,000,000 cells) were injected ex vivo in pig knees, to mimic a clinically relevant sized model. Furthermore, SPIO-labeled cells (10,000 - 1,000,000 per 75 μl) were seeded in cartilage defects in vitro. Scanning was performed on a clinical 3.0 T MRI scanner.
SPIO re-uptake: To study possible SPIO re-uptake by synovial cells, viable and dead GFP-SPIO double-labeled chondrocytes were seeded on human synovium explants. After co-culturing for five days, samples were harvested and analyzed using fluorescence- and light microscopy.

Results: *Safety:* SPIO labeling resulted in labeling efficiencies of ± 95% and did not impair cell viability or subsequent cell activity at any dose. SPIO-labeled hBMSCs produced amounts of glycosaminoglycan and collagen type II comparable to unlabeled control cells.
Intra-articular imaging: All SPIO-labeled cell dosages, both intra-articularly injected and cells seeded in cartilage defects, were visualized by MRI (Fig. 1). Cell-dose dependent signal voids were observed, and cells could be clearly differentiated from anatomical structures. SPIO-labeled cells seeded in cartilage defects could be quantified using a T2* mapping MRI technique.
SPIO re-uptake: GFP^+-$SPIO^+$ cells, indicating originally seeded cells, were seen in samples containing live cells. GFP^--$SPIO^+$ cells, indicating SPIO re-uptake by synovial cells, were found in samples containing dead cells.



Conclusions: hBMSC labeling with SPIO particles is feasible, without leading to negative effects on cell viability, subsequent metabolic cell activity or chondrogenic differentiation. SPIO-labeled cells can be visualized intra-articularly by MRI and quantified when seeded in a cartilage defect. Although possible SPIO re-uptake by host cells has to be taken into account, we showed promising results for the use of SPIO labeling for cell tracking in clinical cartilage repair. This approach provides the extra advantage to simultaneously track cells and evaluate cartilage repair in one MRI session.

164

CHONDROGENIC POTENTIAL OF SUBPOPULATIONS OF CELLS EXPRESSING MESENCHYMAL STEM CELL MARKERS DERIVED FROM HUMAN SYNOVIAL MEMBRANES

M.C. Arufe[1,2], A. De la Fuente[1], S. Díaz[1,2], I. Fuentes[1,2], F.J. De Toro[1,2], F.J. Blanco[1,3]
[1]Osteoarticular and Aging Res. Lab. Cellular Therapy Unit. Ciber-BBN. INIBIC-Complejo Hosp. Univ. A Coruña, A Coruña, Spain; [2]Dept. Med. Area of Anatomy and Human Embryology. Fac. Hlth.Sci. Univ. of A Coruña, A Coruña, Spain; [3]Cathedra BIOIBERICA of Cell Therapy Univ. of A Coruña, A Coruña, Spain

Purpose: Synovial membrane mesenchymal stem cells (MSCs) have been demonstrated to be a good source of cells for the study of cartilage tissue engineering. Multiple stem cells markers have been found by flow cytometry and immunofluorescence in MSCs from human synovial membrane pools. In this study we analyzed the chondrogenic potential of subpopulations of MSCs derived from human synovial membranes enriched for CD73, CD106 and CD271 markers.

Methods: Subpopulations of human synovial membrane MSCs enriched for CD73, CD106 and CD271 markers were isolated using a cytometry sorter and characterized by flow cytometry for MSC markers. The expression of Sox9, Nanog and Runx2 genes by these cells was measured by reverse transcriptase-polymerase chain reaction. The chondrogenesis of each subpopulation was assessed by culturing the cells in a defined medium to produce spontaneous spheroid formation and differentiation towards chondrocyte-like cells. The examination of the spheroids by histological and immunohistochemical analyses for collagen type II (COL2), aggrecan, collagen type I (COL1), metalloprotease 13 (MMP13) and collagen type X (COLX) levels were performed to assess their chondrogenesis capacity. The adipogenesis and osteogenesis potential of each subpopulation was determined using commercial media; the resulting cells were stained with oil red O or red alizarin to test the degree of differentiation.

Results: The subpopulations had different profiles of cells positive for the MSC markers CD44, CD69, CD73, CD90 and CD105 and showed different expression levels of the genes Sox9, Nanog, Runx2 involved in chondrogenesis, undifferentiation and osteoblastogenesis, respectively. Immunohistochemical analysis demonstrated that COL1, COL2, COLX, MMP13 and aggrecan were expressed in the spheroids as soon as 14 days of culture. The $CD271^+$ subpopulation expressed the highest levels of COL2 staining

compared to the other subpopulations. CD105 and Runx2 were shown by immunohistochemistry and genetic analysis to have significantly higher expression CD271+ subpopulation than the other subpopulations.
Conclusions: Spheroids formed from CD271-enriched and CD73-enriched MSCs from normal human synovial membranes mimic the native cartilage extracellular matrix more closely than $CD106^+$ MSCs and are possible candidates for use in cartilage tissue engineering. Both cell types have potential for promoting the differentiation of MSCs into chondrocytes, presenting new possibilities for achieving intrinsic cartilage repair.

165

DIRECT RAAV-MEDIATED IGF-I OVEREXPRESSION ENHANCES ARTICULAR CARTILAGE REPAIR IN VIVO

M. Cucchiarini[1], M.D. Menger[2], D. Kohn[1], S.B. Trippel[3], E.F. Terwilliger[4], H. Madry[1]
[1]Inst. for Experimental Orthopaedics, Homburg/Saar, Germany; [2]Dept. of Clinical and Experimental Surgery, Homburg/Saar, Germany; [3]Dept. of Orthopaedic Surgery, Indiana University, Indianapolis, IN; [4]Div. of Experimental Med., Harvard Institutes of Medicine, BIDMC, Boston, MA

Purpose: Therapeutic gene transfer might be a means to enhance the reparative activities in articular cartilage lesions. We previously reported that recombinant adeno-associated viral (rAAV) vectors are capable of delivering transgene sequences to articular cartilage defects in experimental models over extended periods of time. In the present study, we tested the hypothesis that efficient and sustained overexpression of IGF-I via direct application of rAAV enhances the healing of osteochondral defects created in the knee joints of rabbits *in vivo*.
Methods: rAAV were packaged, purified by dialysis, and titrated by real-time PCR. rAAV-*lacZ* carries the *E. coli* beta-galactosidase (β-gal) marker gene (*lacZ*) controlled by the CMV-IE promoter/enhancer. A human insulin-like growth factor-I (hIGF-I) cDNA was cloned in rAAV-*lacZ* instead of *lacZ* to produce rAAV-hIGF-I. Two osteochondral defects (3.2-mm in diameter) were created in each patellar groove of Chinchilla bastard rabbits (n = 8). Each animal received alternatively 10 μ l rAAV-hIGF-I per defect on one knee (IGF-I-treated defects) and 10 μl rAAV-*lacZ* per defect on the contralateral knee (control defects). At 3 weeks post operation, cartilage repair was assessed based on safranin O/hematoxylin eosin-stained sections using a histological grading system. A total of 145 paraffin-embedded sections (5 μm) were scored independently by two individuals that were blinded with respect to the treatment. Points for each category and total score were compared between the groups using a mixed general linear model with repeated-measures analysis of variance. Indirect immunohistochemical staining was also performed to detect β-gal, hIGF-I, and type-II collagen. The DNA, proteoglycan, and type-II collagen contents of the repair tissue within the defects were measured using Hoechst 33258, by binding to DMMB dye, and by ELISA, respectively. Data are expressed as mean ± SD. The t-test and the Mann-Whitney Rank Sum Test were employed where appropriate.

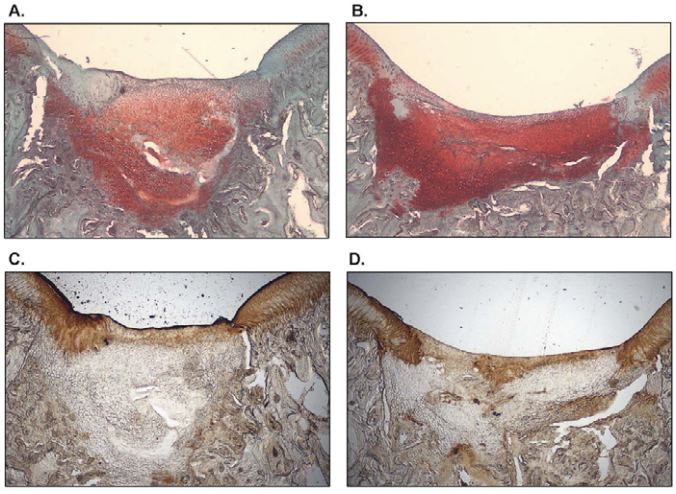


Figure 1. Safranin O staining (A, B) and type-II collagen immunoreactivity (C, D) in osteochondral defects 3 weeks after vector application (A, C: rAAV-lacZ; B, D: rAAV-hIGF-I). Magnification ×2.

Results: Following direct application of the vectors *in vivo*, there were no signs of synovitis, adhesions, or adverse reactions, and no macroscopically descriptive differences between the IGF-I-treated and control knees. β-gal activity was restricted to the control defects, whereas IGF-I expression was present only in the IGF-I-treated defects. After 3 weeks, enhanced tissue healing was observed in the IGF-I-treated defects (Fig. 1). Improved individual parameter scores were observed for defect filling, integration, matrix staining, cellular morphology, defect and surface architecture, new subchondral bone formation (all $P<0.001$) and tidemark ($P<0.01$) of the IGF-I-treated defects, with also a significantly improved total score vis a vis control treatment ($P<0.001$). Immunoreactivity to type-II collagen was more intense and regular in the IGF-I-treated defects (Fig. 1). Biochemical analyses performed on the repair tissue from the defects revealed that treatment with rAAV-hIGF-I promoted a significant increase in the DNA (3.2-fold; $P<0.001$), proteoglycan (1.2-fold; $P = 0.01$), and type-II collagen contents (2.8-fold; $P<0.001$).
Conclusions: The data indicate that IGF-I can be overexpressed in osteochondral defects *in vivo* via rAAV transduction, leading to the production of a recombinant IGF-I factor that is capable of significantly improving the healing of the defects, stimulating both cell proliferation and extracellular matrix synthesis. The results suggest that therapeutic rAAV may have value in enhancing cartilage repair by application to sites of cartilage damage. Further studies are required to evaluate the long-term properties of the repair tissue.

166

CELLULAR AND MOLECULAR CHARACTERISTICS OF CHONDROCYTES DERIVED FROM PATIENTS WITH OSTEOARTHRITIS REVEAL THEIR APPLICABILITY IN MATRIX-ASSISTED AUTOLOGOUS CHONDROCYTE TRANSPLANTATION

J. Ringe[1], T. Dehne[1], C. Karlsson[2], M. Brittberg[3], A. Lindahl[2], M. Sittinger[1]
[1]Charité-Univ. Med. Berlin, Tissue Engineering Lab., Dept. of Rheumatology & Berlin-Brandenburg Ctr. for Regenerative Med., Berlin, Germany; [2]Sahlgrenska Univ. Hosp., Inst. of Lab. Med., Dept. of Clinical Chemistry and Transfusion Med., Gothenburg, Sweden; [3]Sahlgrenska Univ. Hosp., Dept. of Orthopaedics, Gothenburg, Sweden

Purpose: Cartilage defects arise primarily due to traumatic or degenerative changes and in the medium to long-term often result in osteoarthritis and total joint replacement. One aim of modern orthopaedics is to avoid TJR. For traumatic and small focal degenerative cartilage defects, in the recent years promising treatment options like lavage, abrasion, osteochondral autograft transfer, microfracture, cell-free implants, autologous chondrocyte transplantation and matrix assisted ACT have been developed. However, these therapies cannot completely arrest OA progression and currently are not applied for the treatment of large OA defects. This is partly due to a lack of knowledge regarding the cellular and molecular characteristics of OA chondrocytes. The extracellular matrix is the functional element in cartilage and its degradation is central in the pathogenetic process in OA. In addition, new formation of ECM is crucial in OA chondrocyte based therapies. Therefore, the aim of our study was to provide a more complete picture of the cellular and molecular alterations in OA cartilage and to analyze cartilage formation by OA-chondrocytes.
Methods: Human articular cartilage biopsies were collected from OA patients as well as healthy normal donors. RNA was isolated from the biopsies and subjected to genome-wide microarray analysis. Important results were verified using qPCR and immunohistochemistry. Furthermore, chondrocytes were harvested from such biopsies applying protocols used for ACT. Their chondrogenic potential was studied in high-density pellet and hyaff-11 cultures by proteoglycan and collagen type II staining. Moreover, chondrocytes cultured in monolayer and hyaff-11 scaffolds were subjected to microarray gene expression profiling. Again, expression data were verified by qPCR.
Results: We detected genes already associated with normal cartilage and alterations in OA cartilage but also detected new candidates not previously associated with this disease. Here, the expression of genes coding for collagens (COL8A2, COL13-15A1) or related to bone formation (CLEC3B, CDH11, GPNMB, CLEC3A, CHST11, MSX1-2) was significantly higher in native OA cartilage than in native ND cartilage. In 3D hyaff-11 cultures, chondrocytes from ND and OA donors secreted comparable amounts of ECM components like proteoglycans and collagen type II. Expression of cartilage marker genes (ACAN, COL2A1, COMP, SOX9) and genes involved in matrix synthesis (BGN, COL9A2, COL11A1) was highly induced in 3D ND and OA